

A Novel Technique for Determining the Calcium-Binding Properties of the Two Domains of  
Calmodulin in the Presence of Target Peptides

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## Abstract

Calmodulin (CaM) is a ubiquitous calcium-binding protein that acts as a switch to regulate more than 500 different proteins. The classic view of CaM posits that it first binds calcium, undergoes a conformational change, and then binds to a target enzyme to alter its enzymatic activity. In order to understand the myriad of protein interactions of CaM with its targets, it is important to understand the calcium sensitivity of CaM in the presence of its targets. To measure the calcium sensitivity of these events, researchers typically utilize steady-state fluorescent techniques. However, CaM contains two different calcium binding domains located at its N-terminal and C-terminal domains. Calcium binding signals of these two domains overlap during a calcium titration, making it difficult to decipher the contribution of either domain to the binding process. To get around this, we developed a novel technique utilizing a stopped-flow apparatus that allows us to differentiate and separate the two domains in time. Stopped-flow measures the kinetics of a reaction and allows us to view the binding process as it occurs at each domain at a moment in time. By performing a sequence of stopped-flow experiments, each with a slightly different calcium concentration, we were able to simulate a steady-state titration, yet beneficially view each domain as a separate, non-overlapping entity. Thus, we can now successfully observe the calcium sensitivity of each CaM domain in the presence of a target, something that was previously very difficult to do. This technique will allow further research into CaM binding, leading to greater insight into the workings of this protein.

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## Chapter 1

When it comes to understanding just how the world works, it is absolutely essential to comprehend the concept of kinetics. In a general sense, kinetics is simply the study of movement; how an “Object” travels from Point A to Point B. Of course, that is only a very simplistic view of kinetics and one which is not indicative of the field as a whole. The reality is that understanding and studying kinetics requires a more nuanced approach. Like most generalized topics, kinetics can be broken down and categorized: researchers may seek to observe the kinetics of a planet in orbit or the differences in movement between three different species. On a microscopic level, scientists also study kinetics, observing reaction rates for chemical processes. In some instances, kinetics is less about pure movement, and more about the rate of a reaction: A binds with B for X seconds before disengaging. Such activity can fall under the subfield of *receptor-ligand kinetics*. In receptor-ligand kinetics, if a researcher determines how long two proteins in the muscle bind, it might help explain a movement deficiency in a patient. Perhaps one of the proteins is currently present in suboptimal quantities and thus cannot properly bind with the other. Research into kinetics can contribute insight into these protein binding complications. Modifying the conditions under which such a process takes place can alter the speed of a kinetic interaction and researchers work to determine what these alterations entail.

Physiologically the study of kinetics is essential. Within the body, many systems are constantly operating and rarely, if ever, do so in an equilibrium state. An excellent example within the body is the heart. In the heart, the cytosolic calcium concentration is constantly rising and falling, inducing a pumping action caused by contraction and relaxation. When a cardio myocyte's sarcolemma becomes depolarized, calcium ions can enter the intracellular compartment and subsequently cause the release of calcium stored in the sarcoplasmic reticulum (1, 2). These ions can then be bound by calmodulin (CaM). CaM is a protein that is highly involved in the kinetics of the cardiac system and the human body as a whole. CaM is a ubiquitous calcium-binding protein and acts to regulate over 500 different proteins. Generally, transient calcium will bind with CaM and once this has occurred, will change the protein's conformation. This conformational change subsequently allows CaM to further bind and interact with a target protein. At some point however, the calcium comes unbound from CaM and leaves the system, whereupon the protein returns to its original shape. In this form, the bound protein can no longer remain attached and comes free. This cycle repeats continuously until interrupted by cessation of life.

As a laboratory in the Department of Physiology and Cell Biology, the Davis Lab has been interested in the mechanics and kinetics of muscle contraction and relaxation, working closely with proteins such as troponin and CaM. The Davis Lab began researching CaM properties several years ago and this thesis evolved out of that work. While CaM is found throughout the body, work centered on its role in the heart, specifically its interaction with several peptides of interest. By learning more about these interactions, it was and is hoped that there can be further developments in creating

genetically modified hybrids, proteins that are able to interact in a way which is more beneficial to the human body. Typical experiments were undertaken to study the binding process, with the general belief that the study would provide information for a thesis focusing on protein characterization. However an unforeseen difficulty arose, leading to the genesis of a new idea for a thesis topic that might also provide some information about the peptides at hand.

**Problem Statement:** To understand the CaM-calcium-target protein binding process, a common formula is utilized:  $k_d = \text{[off]} / \text{[on]}$ . In this equation,  $k_d$  is known as the “dissociation constant” while off and on represent, respectively, the off-rate and on-rate for calcium binding. That is, the rate at which calcium disengages and engages to the CaM protein. The dissociation constant itself is a value that represents the tendency for a complex to come apart into separate units. The  $k_d$  is a unique value and differs depending on what additional proteins or ions besides CaM and calcium are part of the complex.

This formula is not exclusive to CaM processes and applies generally to protein kinetics. For a researcher to characterize the protein, he or she must first collect information on two of the variables, the  $k_d$  and off-rate and use this data to solve for the on-rate. The variables can be determined through two techniques, titration and stopped-flow. Titration is used to measure the affinity between complex members while stopped-flow measures the rate at which a complex engages or disengages.

In general, titration is a straightforward procedure. Substance A is titrated into Substance B and the substances will then unite together. Progressing through the titration involves slowly increasing the amount of Substance A present in the system until

saturation is eventually achieved. As the concentration of A increases, the change is monitored via fluorescent emission, typically made possible by the presence of either a fluorescent amino acid such as tryptophan, or through an artificial construct. The data is then used to create a curve displaying the effect that increasing the A's concentration has on the system's fluorescent emittance. From this curve, one can extrapolate the data to generate the dissociation constant.

What makes a titration involving CaM more challenging than normal involves the presence of more than one binding event. While binding may occur at only one location for many proteins, calcium ions bind to CaM in *two* locations, two ions per location. This ultimately leads to a net total of four  $\text{Ca}^{2+}$  ions. CaM has two different domains, known as N and C, and each one can bind with calcium ions (See Figures 1 and 2.) This on its own would not be problematic, provided that the binding affinities do not overlap or interfere with one another. Such a scenario would then allow extrapolation of each domain's unique  $k_d$ . Unfortunately this is not always the case with CaM.

When the data collected from a CaM titration is graphed out, it reveals a continuous curve. That in itself may seem benign, but since CaM is known to have multiple binding events, such a curve creates a problem. Two binding events are known to happen, but with the overlap, it is impossible to determine just where the C-Domain begins and ends, as well as where the N-Domain begins and ends. Naturally, this makes it impossible to accurately determine the  $k_d$  for the proteins of interest interacting with CaM and thus makes characterizing these proteins and their CaM involvement impossible as well. While the off-rate may be calculated, the on-rate is still unknown and the affinity is

also an unknown, creating the problem of too many variables. This comes about because with multiple unknowns, there are literally infinite solutions to the equation.

What is needed here is a technique that will allow the  $k_d$  to be established, taking into account the presence of the multiple domains and binding events. Should such a technique be successfully developed, it will allow for the characterization of CaM with calcium in the presence of various target peptides. Moreover, such a technique will be a valuable asset to any other work that involves multiple binding events, regardless of the presence of CaM.

**Literature Review: *Calmodulin***- When discussing CaM, it is beneficial to go into additional detail about the protein itself. CaM is only one of a variety of intracellular  $\text{Ca}^{2+}$ -binding proteins. Specifically, CaM falls under a subcategory of proteins that couple the signal from the calcium to effect changes on a cellular and biochemical level. It has already been noted that CaM is a highly prevalent protein within the human body, allowing it the ability to bind to a variety of proteins. Common examples include protein kinases, phosphatases, nitric oxide synthases, and calcium-extruding pumps (3). The CaM protein itself is a small regulatory protein of 148 amino acid residues, and features 4 structural motifs of a design termed “E-F Hand.” (Figure 3). The E-F Hand motif (Figure 3) features a helix which is followed by a loop, the site of calcium coordination, and then a second helix. Taken together, the arrangement appears similar to the thumb and first two fingers of a hand, hence the name E-F Hand (4). The four E-F Hands are divided into two groups of two with each group being either the N-Terminal or C-Terminal of CaM. Another way of viewing CaM is to think of it as a “dumbbell” with the N and C domains

connected together by a helical “hinge.” As seen in Figure 2-C, when an enzyme or peptide binds with CaM, the hinge swings around the target like a door closing.

E-F Hand Proteins have the ability to respond to calcium in two different ways. The first group of proteins binds to  $\text{Ca}^{2+}$  but refrains from conformational change, acting more as a  $\text{Ca}^{2+}$  buffer. The second group, of which CaM is a part, actually undergoes a change in conformation brought about by the presence of  $\text{Ca}^{2+}$ . This change differs from protein to protein. In the case of CaM, it is interesting to note that each domain exhibits different behavior depending on the environment of the cell. When  $\text{Ca}^{2+}$  is absent, the N-terminal will adopt a “closed” conformation, packing together the helices from both E-F hands while the C-terminal adopts what can be described as a “semi-open” conformation. This is done to bury hydrophobic regions of the protein. However when the calcium concentration transiently rises and calcium ions are bound to CaM, there are changes in each domain, producing “open” conformations. This rearrangement exposes CaM’s regions of hydrophobicity, allowing for further binding of the CaM complex to other proteins (4). Interestingly, CaM has the ability to bind to many different proteins even though each has a unique CaM-binding region. The enzymes have a basic, hydrophobic binding region which is able to favorably interact with CaM’s own hydrophobic, methionine-heavy region (3). This variability is what makes CaM so useful throughout the body.

*Calmodulin Fluorescence:* It was previously noted that one way to measure a titration was through changes in the emitted fluorescence of a protein or protein complex. Although this is certainly not the only way to conduct a titration, it was be the method utilized in this thesis. In nature, this is made possible through the intrinsic presence of

one of three fluorescent amino acids: tryptophan, tyrosine, and phenylalanine. Generally, the strongest fluorescence is emitted from tryptophan while the latter two amino acids emit weaker, but still significant amounts of fluorescence. Researchers can use this fluorescence to monitor and observe changes in a protein during a titration. CaM itself has eight phenylalanine residues, one tyrosine residue, and no tryptophan residues (5). Fluorescence monitoring complications can arise when a protein has too many fluorescent amino acids. For example, with eight phenylalanines, it could be a very difficult signal to track. This difficulty might occur for several reasons. Multiple fluorescent amino acids might monitor different  $K_d$  values, different dissociation rates, or even report completely unrelated events in the protein. However, since CaM only has one tyrosine residue, one can monitor a titration by way of change in the tyrosine fluorescence. This is a useful strategy, however everything changes when a protein is observed in conjunction with another protein; the other protein could likewise have a variety of fluorescent amino acids too. All of the fluorescence makes it hard to get a proper signal. This tends to prevent intrinsic fluorescence from being used to monitor CaM and its protein interactions.

As a result of this problem, last year a colleague in my lab, Ben Jones worked to engineer a CaM with fluorescence that is non-native in design. This is done by a way of a fluorophore that covalently links to the protein of interest (5). Jones was able to substitute a cysteine in place of a threonine at residue position 5, and then covalently link a fluorescent probe, IAANS, to the cysteine. In the natural state, CaM actually contains no cysteine residues. The resulting T5C CaM performed similarly to the wild-type protein in

the presence of various peptides and could thus be used for future studies, including this one.

*Calmodulin-Calcium Binding:* The  $\text{Ca}^{2+}$ /CaM binding process will occur regardless of the presence of proteins and peptides; however in the presence of these target peptides, the affinity that CaM has for calcium is drastically changed. This arises primarily due to the EF-hand nature of CaM. With the EF-hand motif, the  $\text{Ca}^{2+}$  binding characteristics of a given site must be tailored or “tuned” to provide the optimal  $\text{Ca}^{2+}$  activation parameters required by a specific biochemical pathway (6). Moreover, the affinity and kinetics have to allow for activation and inactivation at the proper  $\text{Ca}^{2+}$  concentration threshold and at the proper rate. Tuning can take two different forms, being either intra-molecular or inter-molecular (7). While Peersen notes that intra-molecular tuning is sufficient for a protein that has only one biochemical role, CaM is a multi-functional protein and has to use additional inter-molecular tuning interactions. As the name implies, this type of tuning comes from interactions between different proteins and helps modulate the binding to calcium, allowing for optimized conditions. CaM is an ideal example of inter-molecular tuning because the presence of target peptides and enzymes has been shown to greatly alter its binding affinity. As just a small example, the macroscopic  $\text{Ca}^{2+}$  affinity of CaM can increase from about 14  $\mu\text{M}$  when a target is absent to 1.1  $\mu\text{M}$  in the presence of skeletal myosin light chain kinase (skMLCK) (8). Although this difference may appear insignificant, 14  $\mu\text{M}$   $\text{Ca}^{2+}$  is typically beyond normal physiological levels, while 1  $\mu\text{M}$  is approximately the concentration for a  $\text{Ca}^{2+}$  transient.

During the process when  $\text{Ca}^{2+}$ /CaM binds with a target, the CaM domains wrap around the target. In doing so, this allows CaM’s N Domain to interact with the target



peptide's C-terminal end, and vice versa. Since each target is different, the interactions themselves are as well, affecting the precise CaM conformation and orientation. Targets also have different levels of binding free energy. This structural and thermodynamic variability is a key factor in the variance of inter-molecular tuning (7).

With past work into CaM kinetics, stopped-flow studies were used much as they were in this thesis. They indicated that the increase in affinity was largely a result of a slower  $\text{Ca}^{2+}$  dissociation. Additionally, flow dialysis was used to observe that target peptides including skMLCK, were able to increase the  $\text{Ca}^{2+}$  affinity of CaM, however these newly increased affinities had a broader range of values as compared to those found with intact target proteins (7).

*Target Peptides:* Calmodulin's primary interaction, alongside calcium ions, is with a variety of proteins. While it does interact with hundreds of proteins, it would be unrealistic to test all of them. Thus four proteins were initially chosen as targets for this study; work with one peptide, neural nitric oxide synthase, was eventually halted due to solubility problems. The three proteins all have important functions within the heart but are also important elsewhere in the body, allowing for possible translational uses of this research. The proteins selected for observation include calcineurin (CaN), myosin light chain kinase (MLCK, the specific type here is called RS-20), and CaM Kinase II (CKII). These proteins all have the advantage of being well-studied in general, which was part of the reasoning behind their selection.

It is further important to note that while three enzymes were selected; the research was conducted using only peptide fragments of the enzymes. The reasoning is due to one

of the fundamentals of physiological research: building up a system. As a system becomes more complex, experiments are harder to conduct; one must account for more variables and more factors in the study. Thus before studying an entire muscle, a scientist will use only filaments. Before we can observe the interaction with an enzyme, it is more appropriate to observe the interaction with the smaller peptide. The collected data will thus stand in as rough approximation for the enzyme until further studies elaborate and detail just how the enzyme itself interacts. Additionally, this simpler system is useful for offering insight into the function of more complex systems.

*Myosin Light Chain Kinase:* One of the peptides selected for this study is known as RS-20 and is a fragment of Myosin Light Chain Kinase (MLCK). MLCK is an enzyme that is heavily involved in muscle contraction; however its specific isoform will vary depending on whether the muscle it contracts is smooth, skeletal, or cardiac muscle. In both cardiac and skeletal muscle, contraction is primarily caused through the presence of troponin; however smooth muscle lacks a troponin complex. Thus, the primary agent for smooth muscle contraction is MLCK, acting in conjunction with CaM. In smooth muscle, increasing calcium levels activate CaM which subsequently bind and activate MLCK. This activated kinase complex can then phosphorylate myosin on serine 19. With this phosphorylation, myosin becomes activated by actin which ultimately results in contraction of smooth muscle via an ATP dependent process (9).

Clinically speaking, smooth muscle contractile activity plays an important role in the body, affecting the vascular system, respiratory system, gastrointestinal system, and genitourinary systems. Contraction of smooth muscle is important both in regulating airflow and blood flow. As the heart itself utilizes smooth vascular muscle for the

bloodstream, problems here are essentially cardiac problems as well. Furthermore, problems with this signaling pathway are known to also be involved in several conditions including traumatic brain injury and pulmonary hypertension (10).

While it has already been noted that the troponin complex plays the main role in  $\text{Ca}^{2+}$  regulated cardiac muscle contraction, cardiac MLCK is also involved and it is believed to modulate the contraction process. Moreover, cardiac MLCK has been shown to be involved in both hypertrophic cardiomyopathy and also in severe heart failure (11). Thus the MLCK protein has ties to the cardiac system specifically, yet also is a general protein of the body, making it a desirable choice for research.

*Calcineurin:* A second peptide studied in this project is calcineurin (CaN), a protein phosphatase found throughout the body, much like CaM. In fact, CaN is a protein that has been conserved across all eukaryotic species, emphasizing the importance it plays in growth and development. In humans, CaN takes part in many cellular processes but perhaps the one which is best studied is the immunological regulation of transcription of the t-cell growth factor interleukin-2. This mechanism is entirely calcium-dependent; when there is an intracellular increase in calcium levels, CaM will bind with CaN. Calcineurin can then remove a phosphoryl group from the nuclear factor of an activated t-cell (NF-ATp), allowing for the transcription of IL-2. IL-2 itself is important in activating Helper T-Cells while also inducing other cytokine production (12). Because of its prominent part in immunology, a great deal of research has also focused on the immunosuppression of CaN. This is often brought about through the use of drugs such as cyclosporine (13).

In addition to its immunologic properties, CaN has been documented to be involved in dozens of processes throughout the body. While it is out of the scope of this thesis to discuss those processes, special attention will be paid to the observed affects of CaN in the cardiac system. Like MLCK, CaN is involved in heart disease, specifically cardiac hypertrophy. Once again working with NF-AT, CaN transduces signals responsible for cardiac morphogenesis and hypertrophy. Disrupting these signals can prevent normal cardiac development resulting in the congestive heart failure (13). On the other hand, if CaN is overexpressed *in vivo*, it may also lead to the same problems. Intriguingly, research has shown that cyclosporin A can ameliorate hypertrophy in a mouse model of heart failure (14). Unfortunately, other studies dealing with cyclosporin A in this way have been inconclusive, leading to speculation that multiple pathways may play a role in hypertrophy (14). By learning more about CaN's affinity for binding with CaM in cardiac muscle, this thesis could provide additional background information for future study, especially in understanding under what  $\text{Ca}^{2+}$  conditions CaN activation might occur.

*Calmodulin Kinase II:* The final peptide used in this thesis project is Calmodulin Kinase II (CKII), a  $\text{Ca}^{2+}$ /CaM-depedent protein kinase, just like MLCK. One way in which CKII differs from MLCK is through its ability to autophosphorylize. Once it has been activated by CaM, CKII can undergo autophosphorylization within its autoinhibitory region. This prevents inhibition by increasing CKII's affinity for CaM, a process known as "CaM Trapping" (15). This allows persistence even after free  $\text{Ca}^{2+}$  levels have returned to a basal state (16). Moreover, even when CaM does dissociate from CKII, CKII can remain active longer.

A common thread between peptides of this study has been their ubiquity throughout the body and CKII is no different. It has been most prominently observed in the neurons, but is also involved with fertilization, osteogenic differentiation, vascular tonal maintenance, and cardiac function. Altered CKII levels or regulatory function can impair learning and alter neuronal pathways. Alzheimer's patients also demonstrate abnormal CKII phosphorylation while research involving genetically modified mice with mutations preventing phosphorylation shows them to be more susceptible to seizures (16).

In the heart, CKII activates many different targets including ryanodine receptors, ion channels which help mediate the release of  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum. It is also involved with many voltage-gated ion channels including T-type  $\text{Ca}^{2+}$  channels, L-type  $\text{Ca}^{2+}$  channels, cardiac  $\text{Na}^{+}$  channels, and  $\text{K}^{+}$  Channels. Overexpression of CKII leads to a greater incidence of arrhythmia, myocardial hypertrophy, left ventricular mechanical dysfunction, and increased mortality, suggesting that CKII plays a role in sudden cardiac death (15). Additional research has shown that by inhibiting CKII, hypertrophy can be greatly decreased, along with cardiac dilation and dysfunction that would normally result from a myocardial infarction (17). It is partly because of this role in cardiomyopathy that our lab selected this protein's peptide, along with its prominent role in calcium regulation.

**Specific Aims:** As shown by the literature review for only three targets, CaM is involved in a great deal of essential processes and its responsibilities range all over the body. However it also should have emerged from the review that one of the areas where CaM-related enzymes seem to experience problems is within the heart, with connections being

uncovered to cardiomyopathy and hypertrophy among other disorders. Thus cardiac research itself is of great importance and this thesis can lay some initial groundwork. The general aim for this thesis is to understand more about the characteristics of CaM and how it relates to the three target peptides of interest. When shaping the aims for this thesis project, the original intent was based on the belief that the titration method would be straightforward and uncomplicated. As already indicated, that was not the case. Therefore a third specific aim was added to this thesis in order to accommodate the changed situation.

**Aim 1: Characterize the ability of target peptides to cause conformational change in calcium-saturated CaM.** The initial step in this thesis is somewhat simplistic but nonetheless necessary. The target peptides need to be tested to ensure that they are able to bind with the CaM and effect changes in conformation. Just as important, it must be confirmed that they do so based upon the presence of calcium. This can best be done by observing the fluorescence emittance in two contrasting situations: one where calcium is present and where calcium is absent. The data from each peptide's respective titrations can be compared. Based on previous research, it is believed that the target peptides do in fact bind to CaM only in the presence of  $\text{Ca}^{2+}$ .

**Aim 2: Characterize the steady state and kinetic properties of calcium binding to CaM in the presence of CaM-targeted peptides.** The second goal of this thesis is to determine CaM's affinity, off-rates, and on-rates for  $\text{Ca}^{2+}$  in the presence of a peptide of interest. This will be done through a combination of steady-state titrations and stopped-flow experiments. The titrations will involve titrating a mixture of CaM and target peptide with calcium. As already noted, freestanding CaM does have an affinity for

calcium. However, the addition of peptides should increase the affinity, but not necessarily with the same magnitude. In the heart, not all calcium transients are the same and it seems logical that some transients might not affect all of the enzymes *en masse*. The more these target peptides' affinities differ, the more likely this scenario would appear to be.

Stopped-flow data will also be collected to determine the dissociation rate of the binding region of CaM and  $\text{Ca}^{2+}$ . As noted earlier, the dissociation, or off-rate, is the second piece of information needed to properly characterize the peptide affects of CaM's  $\text{Ca}^{2+}$  binding properties. Together with the on-rate, this helps determine the affinity that  $\text{Ca}^{2+}$ /CaM has for a target peptide. While the on-rate itself can be experimentally determined, it is a rather convoluted process, so the off-rate will be combined with the affinity determined from the titrations, establishing an on-rate. In essence, the rate of CaM binding and dissociation may ultimately determine the activation and inactivation profile of the enzyme, although if there are other events present, this relationship may not be entirely accurate.  $\text{Ca}^{2+}$  plays a key role in this process as it is  $\text{Ca}^{2+}$  binding that starts the entire chain of events leading to target activation.

### **Aim 3: Develop an alternative method for determining the $\text{Ca}^{2+}$ Affinity of CaM.**

While titrations with CaM and the target peptides could be successfully carried out, problems occurred when trying to deconvolute the fluorescent signal. The primary problem as noted earlier was that it was difficult to determine and divide the N and C terminal binding of CaM from each other. Therefore, a new technique needs to be developed that will allow for separation of the domains, allowing for the signal to be deconvoluted and separate affinities for the N and C terminals to be calculated.

**Hypothesis:** It is the third aim which will be the primary focus of this thesis. Stopped-flow is generally a way of observing molecular kinetics; however given the intricate relationship between the steady-state, observed in titrations, and kinetics it was believed there might be a way to utilize it in regards to the steady-state as well. In this case, by performing a series of kinetic experiments and increasing the calcium concentration over the runs, a titration can be simulated in a comparable fashion to that of an actual titration. In essence, the kinetic experiments begin and end in a steady-state. If we can define these conditions in terms of the protein and  $[Ca^{2+}]$ , then the steady-state titration can be recreated. Before mixing in stopped-flow, we can establish a clear pCa, occupied by the  $Ca^{2+}/CaM$  complex in a steady state. Through stopped-flow, the  $Ca^{2+}$  is pulled off and we can measure the amplitude of change in the complex's fluorescence brought about by the departing  $Ca^{2+}$ . This will allow the affinity data to be collected while also keeping in mind the separation between the domains. The collected data will be able to be verified by reconstructing a curve approximate to what is seen in a titration.



## Chapter 2

**Methodology:** A variety of techniques was used early in the process to prepare the proteins and buffer used in this thesis. Because that was work done prior to the onset of this thesis and also for the sake of brevity, much of that earlier information has been condensed.

*Protein Isolation/Purification:* The T5C CaM used for this research was previously designed by Ben Jones, a student in the Davis Laboratory (5). Through his research T5C CaM was identified as the best possible candidate to replicate the effects of wild-type (WT) CaM while still allowing for the presence of a fluorescent probe. After designing this recombinant CaM and comparing it against several other designs, this one was chosen for replication via plasmid transfection. The plasmids containing mammalian recombinant calmodulin protein were then expressed in a BL21(DE3)pLysS strain of *E. Coli* in LB Medium, a strain which is designed to facilitate mutant DNA expression. This was also aided by the presence of a transcription factor, IPTG, which activates the *lac* operon, a CaM promoter. Following this cellular growth, the CaM was purified by standard laboratory procedures including sonification, centrifugation, precipitation, and column chromatography.

*Fluorescent Labeling:* Following the preparation of the T5C CaM, the next goal was to label the protein with a fluorophore. The probe chosen was 2-(4'-(iodoacetamido)anilino)naphthalene-6-sulfonic acid, also known as IAANS. IAANS was

selected as the probe for a variety of reasons, the first being that it was able to react to the cysteine residue itself. Moreover, IAANS is known to be an environmentally sensitive fluorophore, and of the various fluorescent probes available, had the best signal-to-noise ratio. As a final factor for consideration, it had a weight that was about equivalent to that of a tryptophan and thus not big and bulky.

To prepare for the labeling, T5C was dialyzed against 50 mM Tris, 6M Urea, 90 mM KCl, and 1 mM EGTA, pH 7.5. Labeling was initiated by the addition of 3-5 mM excess of IAANS which was able to react with the thiol group of the added cysteine residue. The labeling reaction was allowed to proceed in the dark for 5 hours with constant shaking at 39.2 °F. The reaction was stopped by addition of 5 mM DTT, and un-reacted IAANS was removed by exhaustive dialysis against 10 mM MOPS, 90 mM KCl, pH 7.0. The resulting solution was then aliquoted out and frozen in a -20 °F freezer for future use.

*Peptides:* Peptides used in this project include Calcineurin, Myosin Light Chain Kinase (RS-20), and Calmodulin Kinase II at stock concentrations of 1 mM. Their respective amino acid sequences can be found in Table 1. They were synthesized by Celtek Peptides. For their use in this thesis, the peptides were purified via dialysis in a buffer of 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) and 150 mM Potassium Chloride (KCl), pH 7 to remove some suspected synthesis contaminants. Of the dialyzed peptides, about 500 µL were diluted to 0.1 mM.

*Steady State Fluorescence:* All steady-state fluorescence measurements were performed using a Perkin-Elmer LS 55 spectrofluorimeter at 20°C. The temperature was maintained

by a circulating water bath. IAANS fluorescence was excited at 330 nm and monitored at 450 nm as microliter amounts of  $\text{CaCl}_2$  were added to 2 mL of each CaM complex. The selected wavelengths correspond specifically to IAANS fluorescence, preventing any endogenous fluorescence from being detected and ensuring that the signal itself is clearly IAANS-based in origin. Unless otherwise noted, the buffer utilized in all experiments was 200 mM MOPS, 150 mM KCl, 2mM EGTA, 1mM DTT, 3 mM  $\text{Mg}^{2+}$ , and a pH 7.0. The idea behind titration is straightforward. Due to the presence of the fluorophore, CaM is constantly emitting “baseline” fluorescence, even in the absence of  $\text{Ca}^{2+}$ . When calcium is added to the mix, the binding will cause a change in conformation. The presence of the peptide also uniquely affects the conformation of CaM. The spectrofluorimeter seeks to measure this change in conformation through the environmentally sensitive fluorophore of IAANS.

How exactly does this work? Imagine a shooting gallery where the shooter aims a water pistol at a row of ten targets. Above each target is a light that flashes once the target has been hit. Now when a person begins shooting, not a single target is lit up but as the shooter progresses, more and more targets are hit and more and more lights go off until all ten lights are flashing. The employee running the range then gives a final tally for the game.

Fluorescence titration works much like this, except that in place of a flashing target, there exists a fluorescent CaM, and instead of only ten targets, there exist millions of CaM proteins. Before calcium is added, fluorescence is still being emitted, like a light that is already on. As the titration progresses and more calcium is added to the mix, more and more CaM is able to complex with the  $\text{Ca}^{2+}$  and the peptide as well. In doing so, the

“target” “flashes;” CaM changes conformation and emits a new level of fluorescence. The spectrofluorimeter is able to observe these changes and assigns them an arbitrary level of fluorescence. The emitted fluorescence changes because more and more of the CaM “targets” are being activated and emitting at that new fluorescence; the contents of the entire test vial become saturated.

Alternatively, imagine that all of the individual CaM are pieces within a large light fixture controlled by a dimmer switch. In the apo-CaM state, the light is at some baseline dimness level; each CaM is dimmed. The presence of  $\text{Ca}^{2+}$  causes binding to begin and as individual CaM bind, they brighten. The light fixture then begins to brighten as a whole because the dimmer switch is turned up, until it is “saturated” and well-lit. This would coincide with CaM being completely saturated by  $\text{Ca}^{2+}$ .

Turning back again to the shooting gallery, at some point all ten targets have been hit and all ten lights are flashing. The shooter can continue to fire away and flood the gallery with water, but no more targets can go off. This is the same for CaM; at some point, the system is saturated with calcium ions and the addition of more will not effect a subsequent change in emittance. In this way, titrations are effective in determining the calcium affinity: by graphing the emittance levels at each  $\text{Ca}^{2+}$  addition, a curve can be prepared and from this curve comes the calculation of affinity.

Initial titration experiments focused on the reactivity of the target peptides in apo-CaM, or CaM lacking calcium. In these experiments, 0.26  $\mu\text{M}$  of CaM was titrated by four consecutive 1.6  $\mu\text{L}$  dosages of the target peptide, resulting in a final concentration of

3.2  $\mu\text{M}$  of total peptide. At this point, 12.19  $\mu\text{L}$  of 0.343 M  $\text{Ca}^{2+}$  was also added resulting in a pCa of 4.

The intent behind this set of experiments was simply to verify that the peptides themselves were reacting with CaM only in the presence of  $\text{Ca}^{2+}$ . For that to be the case there should be no observed change in the emitted fluorescence, indicative of no conformational change until the additional presence of  $\text{Ca}^{2+}$ .

The second round of titrations undertaken flips the titrant around with calcium serving as the substance of addition. In the standard spectrofluorimeter vial are both 0.5  $\mu\text{M}$  CaM and 2.5  $\mu\text{M}$  of peptide, a saturating [peptide]. Beginning with a pCa greater than 9 (the absence of  $\text{Ca}^{2+}$ ), additional amounts of calcium were added, decreasing the pCa from 9 to 4. In between the start and endpoint, 12 additional pCa levels were tested in a range that could suitably generate a titration curve. The additional calcium stocks fluxuated between either 0.0343 M or 0.343 M in concentration depending on what needed to be added and to ensure the amount added minimally diluted the sample. Note that the pCa values were determined by the software program EGCA02 developed by Robertson and Potter (18).

This set of titrations was designed to determine the  $\text{Ca}^{2+}$  affinity held by the CaM-Peptide complexes. The collected data was graphed using the Figure P program where the  $\text{Ca}^{2+}$  sensitivity was calculated and reported as an affinity constant  $k_d$ , representing a mean of 3-6 titrations depending on the peptide. The data was also then fit with a single Hill equation to determine the steepness of the curve. An example of the equation is found with Table 2.

*Stopped-flow Fluorescence:* Kinetic information, specifically  $\text{Ca}^{2+}$  dissociation rates  $k_{\text{off}}$ , was measured using an Applied Photophysics Ltd. (Leatherhead, U.K.) model SX.18 MV stopped-flow instrument at 20°C. The IAANS emission was monitored through an Oriel 510 nm broad band-pass interference filter, with excitation at 330 nm. Each  $k_{\text{off}}$  represents an average of three traces, and was fit with either a single or double exponential equation. The buffer used (unless noted otherwise) in all stopped-flow experiments was 200 mM MOPS, 150 mM KCl, 2mM EGTA, 3 mM  $\text{MgCl}_2$ , 1 mM DTT, pH 7.0.

Stopped-flow works through a mixing process. Two syringes in the apparatus itself are rapidly mixed together with a dead time of 1.4 ms. This allows for the measurement of rates at which  $\text{Ca}^{2+}$  dissociates from CaM in the presence of target peptides. The first syringe of the apparatus contains 1  $\mu\text{M}$  of CaM, 5  $\mu\text{M}$  of target peptide, and however much  $\text{Ca}^{2+}$  is currently being tested. The second syringe contains only buffer and 8 additional mM of EGTA which is the key to the dissociation process. That is because EGTA is a calcium chelator, able to complex to the  $\text{Ca}^{2+}$  and prevent their association with the CaM complex. Moreover, EGTA demonstrates a higher affinity for the  $\text{Ca}^{2+}$  and prevents any re-association. After the mixing is complete, EGTA is thus able to rapidly chelate any free  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  also dissociates from the CaM protein. Pulling off  $\text{Ca}^{2+}$  causes a conformation reversion, altering the emitted fluorescent signal from CaM. This signal can then be used to generate a rate-dissociation curve for CaM, showing the process in which  $\text{Ca}^{2+}$  dissociates from both the N and C domains of CaM. The rate can then be fitted and quantified, allowing for comparisons to be made between different peptides' effects on CaM's rate of calcium dissociation. Identifying which part

of the curve is which domain is made possible through previous research. The N and C domains of CaM can actually be fragmented from CaM as a whole and studied individually. Moreover, the C Domain has a fluorescent tyrosine residue that can allow the C domain to be monitored and characterized.

When it became clear that the data gathered in the titration could not be used to properly characterize the  $\text{Ca}^{2+}$  complex, stopped-flow was used as a substitute. With stopped-flow, a series of runs were performed over the same range of pCa as in the titrations. The pCa values ranged from 9 to 4 before mixing occurred. Each run the pCa was lowered a bit more allowing for a slightly different dissociation curve to be presented. This was because as the  $\text{Ca}^{2+}$ /CaM/peptide complex's  $[\text{Ca}^{2+}]$  was increasing, the recorded amplitude was changing. After concluding the shooting, calculations are made for the amplitudes of both the N and C domains respective fluorescence levels. These amplitudes are tabulated and used to generate curves showing the N and C domain changes in fluorescence. Note that the curves utilized exponential equations to provide for a proper fit. The data can also be fit with a Hill equation to determine the steepness of the curve. An example of the equation is found with Table 2. By then combining the domain amplitudes together, as seen with RS-20 in Figure 14, one can observe the fluorescence change for the entire CaM Complex as a whole, thus mimicking what appears during a typical steady-state titration and thus being able to be used as a reliable stand-in for titration, especially in instances where both domains overlap in their  $\text{Ca}^{2+}$  dependent fluorescence changes.

**Statistical Analysis:** For the research conducted in this thesis, the program Minitab for Windows by Minitab Inc. was used to calculate the mean and standard error for affinities,

Hill Coefficients, and dissociation rates. Tables 2 and 3 offer complete lists of all calculated data.



### Chapter 3

**Results:** In order to measure the  $\text{Ca}^{2+}$  binding properties of CaM in the presence of the peptides, it was first important to know that the peptides bound to CaM and also at which concentration they saturated the CaM. Figure 5 shows that the peptides do not bind or interact with CaM in the absence of  $\text{Ca}^{2+}$ . However in the presence of saturating  $\text{Ca}^{2+}$ , at a pCa of 4, the addition of each peptide decreased the level of IAANS fluorescence. This resulted in affinity values for each target peptide: CKII had an affinity of  $200 \pm 7$  nM, similar to MLCK which had an affinity of  $150 \pm 7$  nM. CaN had a much lower affinity of  $700 \pm 30$  nM. Each titration was repeated at least three times to verify the results.

Figure 6 displays the steady-state titrations which were performed to determine the  $\text{Ca}^{2+}$  affinity of CaM in the presence of target peptides. Notice that the shapes of the  $\text{Ca}^{2+}$  titration curves are multiphasic; this is most noticeable on the titration curve with CKII. This data suggests that there are at least two events occurring during the titrations. It is too difficult to discern these events from the steady-state titrations and thus the stopped-flow method was created which will be described later.

First, stopped-flow experiments were performed to determine the  $\text{Ca}^{2+}$  off-rates from CaM for each of the peptides; this can be observed in Figures 7-9. Each figure displays an individual dissociation run for each of the target peptides and also details each peptide's effect on the  $\text{Ca}^{2+}$  dissociation rates, one rate for each domain of CaM. CaN only has evidence of N terminal kinetics. CKII and RS-20 each show evidence of

two domains, N and C, being affected by the calcium dissociation with a rise in fluorescence being observed in the N-Domain followed by a decline in fluorescence in the C-Domain. In terms of  $\text{Ca}^{2+}$  dissociation for the N-Domain, CaN had the greatest rate while CKII and RS-20 had roughly half the rate of CaN (see Table 3 for comparison.) Their respective C terminals had even slower rates of  $\text{Ca}^{2+}$  dissociation, occurring below 2/s. Thus the two binding processes can be separated in time.

While kinetic data was collected in the standard way via stopped-flow, this technique was also used to gather affinity data akin to that collected from a steady-state titration; an example of this technique is displayed for CKII in Figure 10. Each target peptide underwent at least three tests for consistency. As the pCa was decreased, the amplitudes of the fluorescence for the N and C domains increased. The amplitude change was then calculated for both the N and C terminals, providing data to construct new affinity curves specific to each domain. As can be seen in Figures 11-13, the curves generally fit well with a Hill equation, with the exception of the C terminal in the presence of RS-20. This seemed to have some additional, as-yet unknown binding event occurring, resulting in a decrease in fluorescence at a pCa below 6. The fitted version only used data up until a pCa of 6.0. With these curves, the  $\text{Ca}^{2+}$  affinities could be tabulated and are displayed along with each curve's Hill Coefficient in each peptide's respective figure. Note that RS-20 appears to have the highest C-terminal affinity at ~430 nM followed by CKII (~501 nM). As previously noted, CaN did not seem to have significant C-Terminal binding (Figure 11). Interestingly, CKII had the lowest N-terminal affinity at ~580 nM followed by RS-20 (~610 nM) and then CaN with ~640 nM (Figures 11-13). All of this data is also collectively displayed in Table 2.

Returning again to the amplitude data collected from stopped-flow, it was now also possible to graph out a stopped-flow curve by combining the C and N terminal amplitude data to compare it to the steady-state titration curve in order to see if the two curves had a similar shape. While the amplitude data was normalized for the individualized domain affinity curves, the raw data was combined together to create a steady-state approximation curve as seen in Figure 14 for MLCK. By combining the N curve amplitude and the C curve amplitude, a final curve is created that is somewhere in between the two separate domain curves. After the N and C amplitudes were combined, this curve was then compared against the original steady-state titration curve. This can be seen for each peptide in Figures 15-17. The two curves are not perfectly overlapping, but the *shapes* of the curves appear to be very similar. This tends to lend credence to the idea that stopped-flow titrations can be an effective substitution for a multiphase steady-state titration.

Using the affinity and off-rate data, the association rate could also be calculated for  $\text{Ca}^{2+}$  in the presence of each peptide. This is seen in Table 3. Note that the  $\text{Ca}^{2+}$  on-rates are much greater than their respective rates of  $\text{Ca}^{2+}$  dissociation. This appears valid; the peptides need to rapidly associate with CaM in the presence of calcium in order to effect activation of targets, but a low rate of dissociation may be desired so that the effects have a longer efficacy time.

## Chapter 4

**Discussion:** When looking at the results, it may make the most sense to consider each peptide individually at first and then look at the entire group. CaN is perhaps a good choice for the first peptide of consideration. As noted, while the other two peptides each had two calculated off-rates, CaN only showed  $\text{Ca}^{2+}$  dissociation for the N domain (see Figure 7). This seems to be supported by the evidence; there seems to only be one clear change in fluorescence emittance, an increase. Moreover, the titration in Figure 6 also presents a fairly standardized curve; that is, it appears to only have a single phase. This may be because as compared to CKII and RS-20, CaM takes a different approach when binding CaN (Figure 4). Why is fluorescence only seen for one domain when both domains of CaM are known to bind CaN? A possible explanation centers on CaM's CaN-binding style. First note that the IAANS fluorophore is located near the beginning of the N domain and thus the C domain has no direct connection to the emittance of fluorescence. For peptides such as CKII and MLCK, this is not necessarily a limitation; when the C domain of CaM binds to these peptides it does so in close proximity to the N-domain of CaM and the fluorescent probe. Thus it is likely that the environmental changes in the C domain affect the IAANS probe, resulting in an additional subsequent fluorescent change. CaN has a different binding style. While CaM wraps itself around the other two target peptides (as depicted in Figure 4,) when binding CaN, the N domain will bind with one peptide while the C domain binds with another CaN peptide entirely. The C domain of a CaM binding CaN is thus far from its partner N domain and too far away

to cause a fluorescent change. The result is that fluorescence is only observed for the N terminal and not the C terminal, hence the lack of C terminal information. CaN-associated CaM also has the lowest  $\text{Ca}^{2+}$  affinity of the three tested peptides,  $640 \pm 17$  nM and also the lowest peptide affinity,  $700 \pm 30$  nM, perhaps due to its unique peptide-binding style.

Next consider CKII, in this case the ideal example of what the stopped-flow titration technique was designed to do. From Figure 6, it is clear something multiphasic was occurring. Figure 8 also clearly showed that two domains were experiencing  $\text{Ca}^{2+}$  dissociation. There is an initial upsurge in fluorescence caused by the N terminal and then  $\text{Ca}^{2+}$  from the C terminal begins to dissociate as well. This would explain why in the titration, an initial increase is seen due to the C terminal binding, but it is overpowered by the large decrease in fluorescence caused by the N terminal binding. When the signal is fully deconvoluted, it is clear that the N and C domains each have unique affinities (Figure 12) but very similar Hill Coefficients and thus similar steepness and cooperativity. When CKII's two domains are reconstructed into a titration, it is similar to the original steady-state titration (Figure 15) if shifted slightly. CKII offers the best evidence that this technique is valid and could be used more definitively in the future.

RS-20 presents an interesting turn of events. Like CKII, it does feature two binding domains, although the second domain's fluorescence is a bit more subtle as seen in Figure 9. There is a net decrease, but just barely. A problem emerges with trying to fit the curves of both the N and C domains: the N terminal fits fine, but the C terminal affinity is thrown off due to an unexpected decrease in the amplitude at high  $[\text{Ca}^{2+}]$ . The most likely explanation is that there is a third event going on somewhere with the entire

Ca<sup>2+</sup>/CaM/RS-20 binding process. Unfortunately, this binding event is currently unknown. What does seem to be clear is that RS-20 has an N terminal affinity that is relatively low compared to CKII and a C terminal affinity that is higher by about 200 nM than the N terminal. This would allow the C terminal to start binding comparatively earlier than the N terminal. When the two domains are recombined to form a titration curve, it appears to have the best similarity among the three target peptides.

RS-20 confusion aside, the results seem to show that the stopped-flow approach to titration is valid. It is admittedly clear that the fit is not perfect, and that is perhaps indicative of future work that should be done to increase the accuracy of this method. Error could have been present in several forms, with human error as a possibility. When calculating volume amounts for the stopped-flow, small errors might have also been introduced. Moreover, the equipment used in this thesis had somewhat different sensitivities, with stopped-flow being less sensitive than the spectrofluorimeter resulting in different accuracies for data collection. Controlling experimental temperature was also difficult in the laboratory environment which sometimes experienced volatile shifts in room temperature. The peptides themselves had to be carefully dialyzed to avoid contaminants, but some insoluble particles may have still been present as they were with the nitric oxide synthase. Nonetheless, it does seem that for binding events involving at least two domains, this could be an effective technique.

**Significance of Research:** Enzymes that bind with CaM perform many different functions within the heart. Problems with these enzymes can result in issues related to contraction and relaxation. For example, it was noted that one enzyme which binds to CaM, CKII, has increased activity in patients with cardiomyopathy, and its expression is

able to alter cardiomyocyte function, causing hypertrophy and eventually heart failure (15). Moreover, research has shown that the inhibition of calmodulin-dependent protein kinase II results in a decrease of extensive myocardial hypertrophy and dilation, lowering the risk of heart disease (3). It was also noted already that both CaN and MLCK play different roles in cardiovascular disease as well. Furthermore, all three of these proteins studied also have important roles to play elsewhere in the body; CaN, for instance, is important in the immunological system. The more research that is obtained about these proteins and the ways in which they activate and act, the bigger the overall benefit will be. Thus, it is beneficial clinically to gain as much insight into these specific proteins as possible.

It is also of benefit to study CaM to a greater degree; CaM truly is an omnipresent protein and has a part to play in many systems within the body. This research is a basic step and a small block of overall CaM research, but still plays a part in progressing the field.

Some of the benefit comes from eventual disease-fighting implications. Part of this thesis aimed at understanding more about the process in which CaM binds  $\text{Ca}^{2+}$ . A goal in the distant future would be to use this data and more refined data that could be collected in the future, to modulate the  $\text{Ca}^{2+}$  binding process in some way. Since many enzymes which are linked to various diseases have an activation pathway stemming from calcium binding, it stands to reason that if we can alter the calcium binding, we can alter the extent of these diseases. Modulation of calcium could allow us to control the extent of subsequent enzymatic activation and the extent of aforementioned cardiac diseases like hypertrophy and myocardial infarction. We might also seek to create CaM mutants that

allow greater activation of a specific enzyme while maintaining the normal activation of the others.

The stopped-flow titration technique itself is also of benefit for research, mostly because it offers a new alternative for future researchers who may run into similar problems as those which were encountered in this thesis. By using the stopped-flow technique, it is possible to work around the affinity overlap caused by having two domains that bind in such relative proximity. If the technique can be improved even more and present even better pseudo-titration curves, it would then be more valuable. It has use for many proteins where multiple binding events are present. For MLCK, the steady-state titration completely masked the occurrence of *three* binding events, something that the stopped-flow technique made clear. This could be a beneficial way to study other proteins as well, revealing additional binding events where it was previously believed to only have one occurring.

Looking ahead, this technique could also be used to study other enzymes and their peptides that bind to CaM. With 500 possible targets, there should be plenty of options. Something involving greater complexity might be to look at expanding the technique to the next stage in the physiological chain: enzymes. Enzymes bring a variety of new challenges, but stopped-flow titration might have a part to play in that setting as well.

In short, this thesis managed to arrive at a few useful conclusions. First, the steady-state titration curves of CaM in the presence of target peptides can be complicated by multiple phases. Evidence suggests that the N and C domains of CaM are the cause of such complications. Through stopped-flow, it is possible to separate the multiple phases



in time. Additionally, by altering the  $[Ca^{2+}]$  at which the stopped-flow experiments were performed,  $Ca^{2+}$  dependent changes in fluorescent amplitude can be observed for each of the CaM domains. This allows for the determination of these domains'  $Ca^{2+}$  binding properties. Essentially, stopped-flow offers a reliable technique to simulate a steady-state titration, especially useful for instances in which traditional titrations are unmanageable and provides new avenues for future research.

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Table 1: Amino Acid Sequences of Target Peptides

Peptides	Amino Acid Sequence
CaN	ARKEVIRNKIRAIGKMARV
MLCK	ARRKWQKTGNAVRAIGRLSS
CKII	MHRQETVDCLKKFNARRKLKGAILTTMLA

Table 2: Peptide Affinity Values

Peptide	Avg. Affinity, X50		Avg. Affinity, nM		K Value		Hill Coefficient	
	N Terminal	C Terminal	N Terminal	C Terminal	N Terminal	C Terminal	N Terminal	C Terminal
CaN	6.195	—	640 ± 17	—	7.7 ± 0.3	—	3.3 ± 0.1	—
CKII	6.234	6.298	580 ± 17	500 ± 14	8.4 ± 0.3	8.9 ± 0.3	3.7 ± 0.1	3.8 ± 0.1
RS-20	6.218	6.372	610 ± 48	430 ± 19	8.0 ± 0.5	7.9 ± 0.7	3.5 ± 0.2	3.4 ± 0.3

*The Hill Equation*

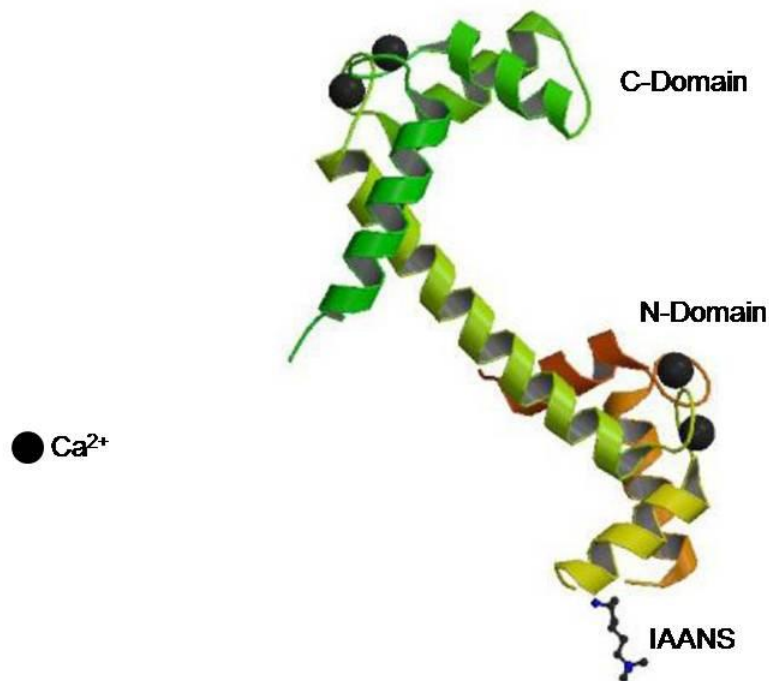
$$\log \left( \frac{\theta}{1 - \theta} \right) = n \log [L] - \log K_d.$$

Table 3: Peptide Association and Dissociation Values

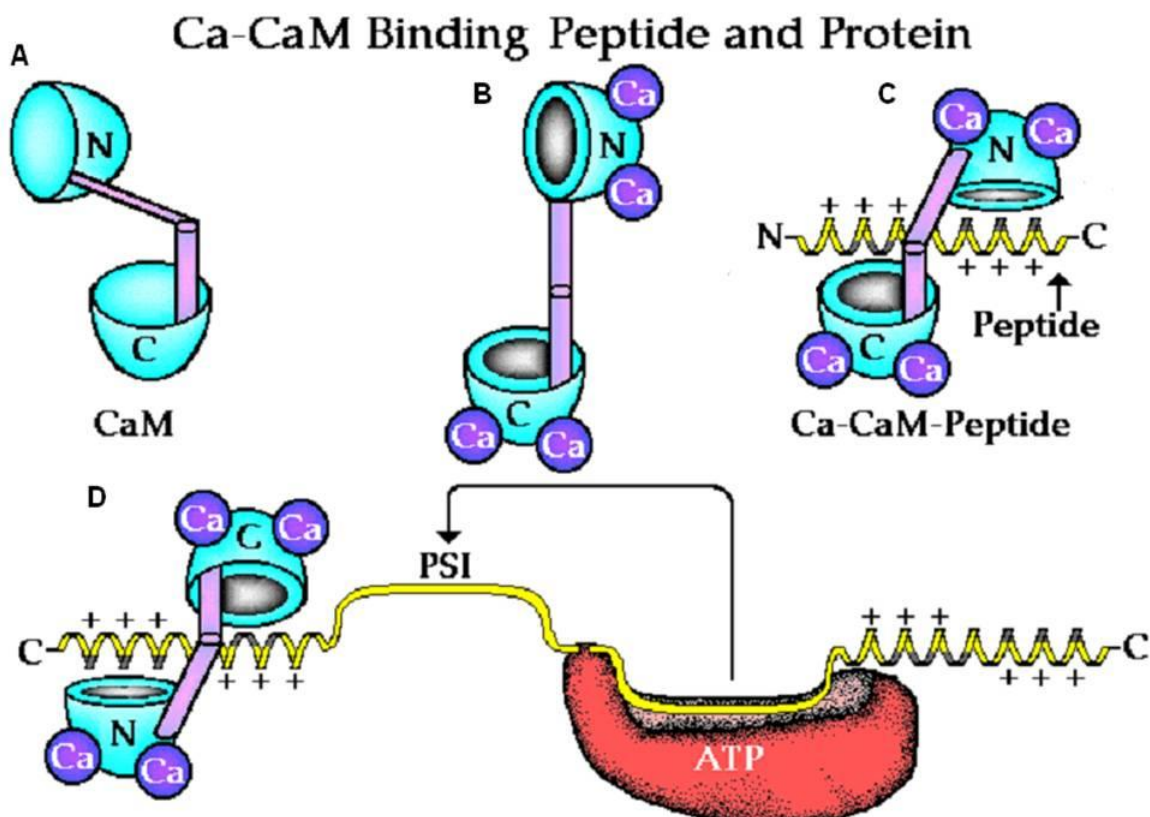
Peptide	Dissociation Rate, [off]		Association Rate, [on]	
	N Terminal	C Terminal	N Terminal	C Terminal
CaN	13.1 ± 0.2	-	20500000 ± 627000	-
CKII	7.3 ± 0.1	1.44 ± 0.01	12600000 ± 407000	2880000 ± 83000
RS-20	6.79 ± 0.03	0.98 ± 0.04	11100000 ± 877000	2280000 ± 137000

Dissociation rates are measured /s while association rates are measured /nM/s

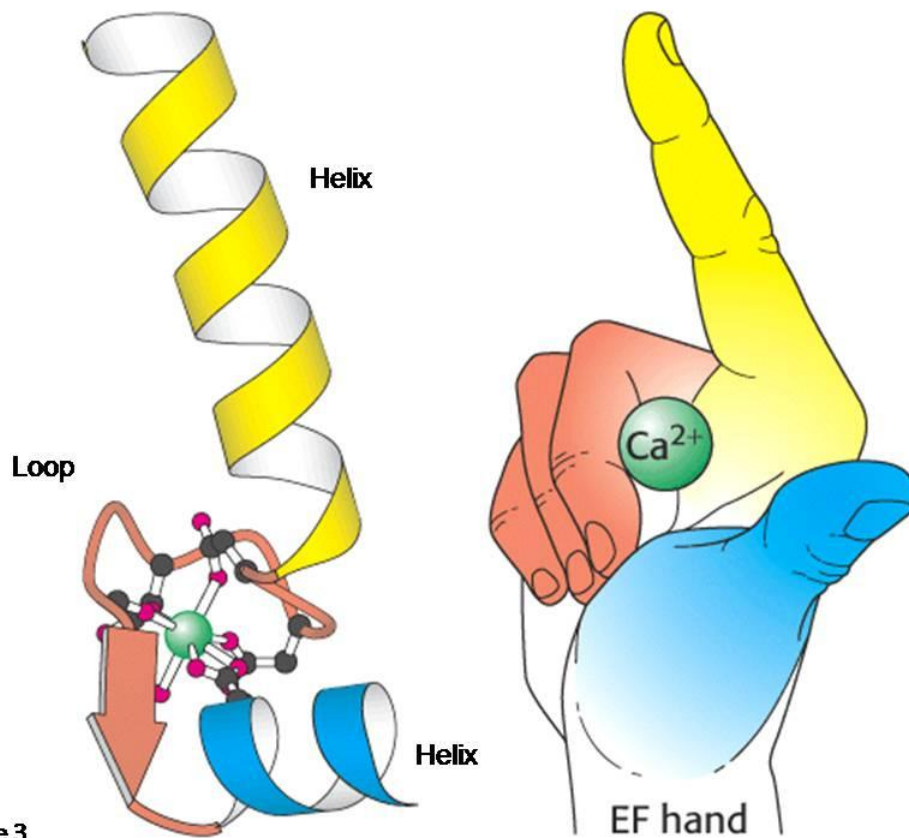
# CrystalStructure of $\text{Ca}^{2+}$ Saturated CaM



**Figure 1** This is the crystal structure of CaM with four bound calcium ions. Note the presence of an attached IAANS Fluorophore. (19)



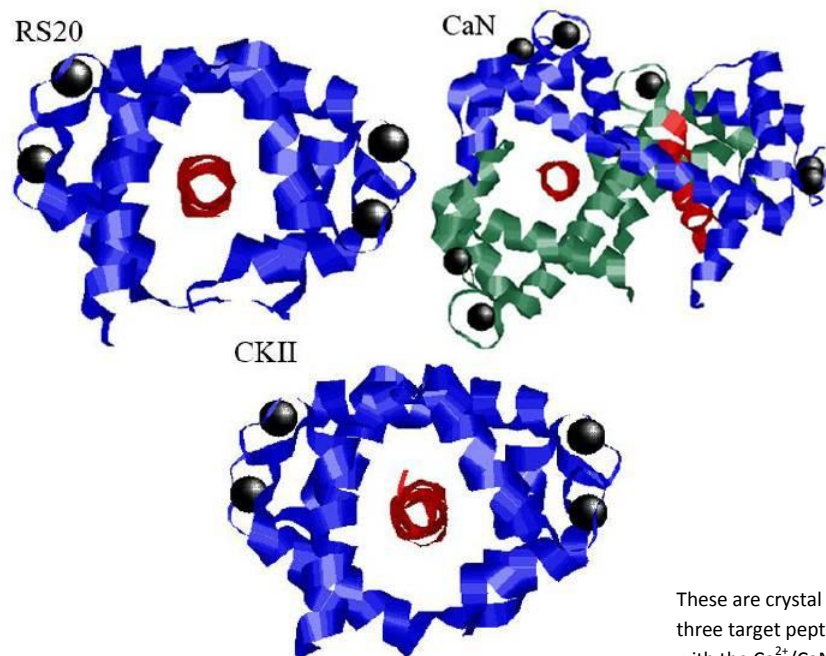
**Figure 2** This is a cartoon Schematic of calmodulin binding calcium and interacting with a target peptide. Also shown is a cartoon schematic of calmodulin activation of a target kinase. Note the binding of calmodulin to its target at a domain adjacent to the pseudo substrate inhibitory (PSI) domain. The binding of CaM causes the release of the PSI domain from the active site, initiating activation.



**Figure 3**

An example of the EF Hand Structure. This involves a helix, followed by a loop, and then a helix. Ligand binding occurs at the loop. Together, the three components resemble an index finger, the other fingers, and the thumb, hence the "hand" shape.

## Structures of Peptides Bound to $\text{Ca}^{2+}$ Saturated CaM



**Figure 4**

These are crystal representations of the three target peptides and their relationship with the  $\text{Ca}^{2+}$ /CaM Complex. Note the similar binding style utilized by CKII and RS-20 (MLCK). Moreover, notice how CaN is differently bound in a process involving the N-Domain of one CaM and the C-Domain of another.



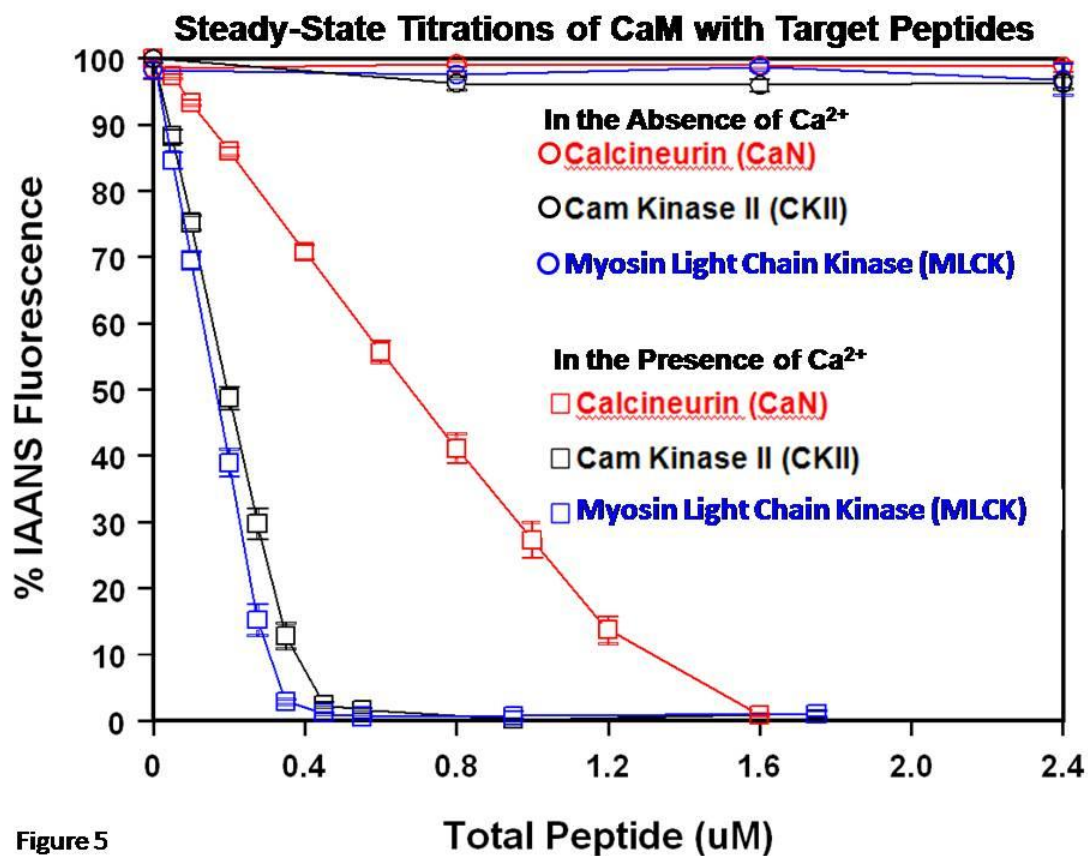


Figure 5

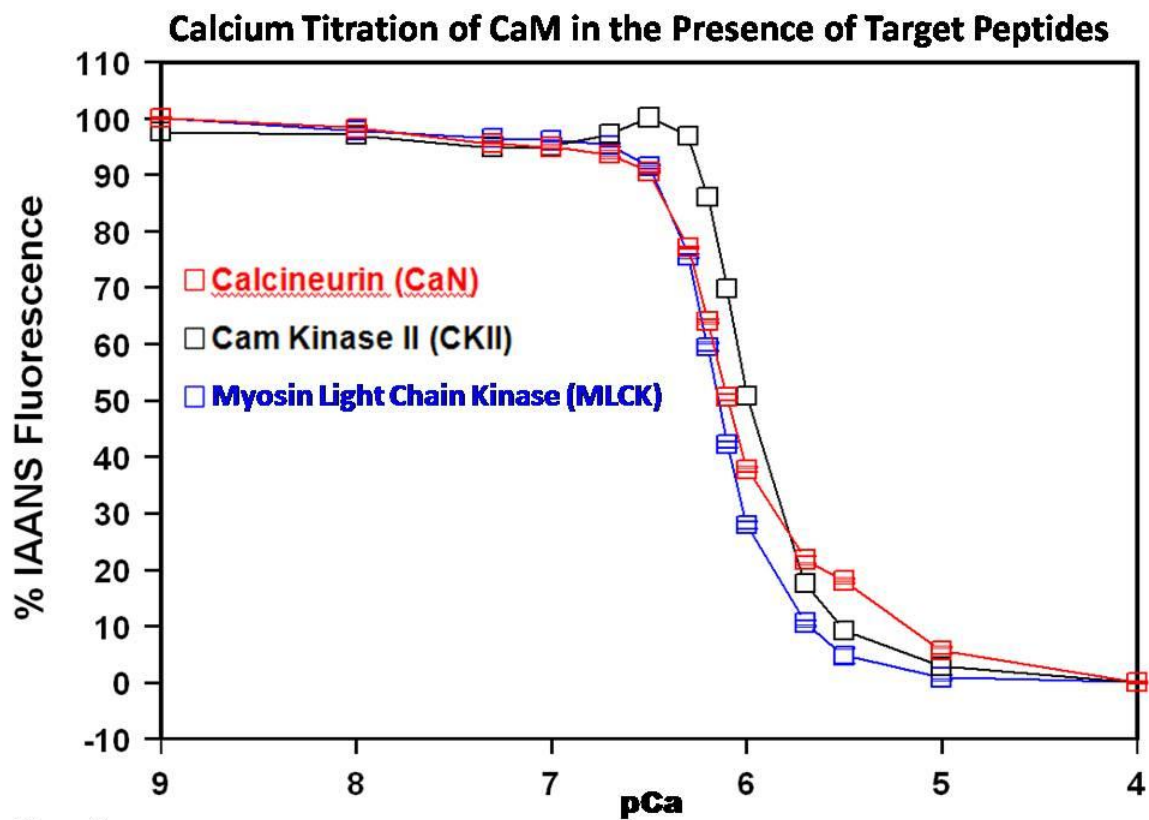


Figure 6

### Calcium Dissociation from CaM in the Presence of CaN Peptide

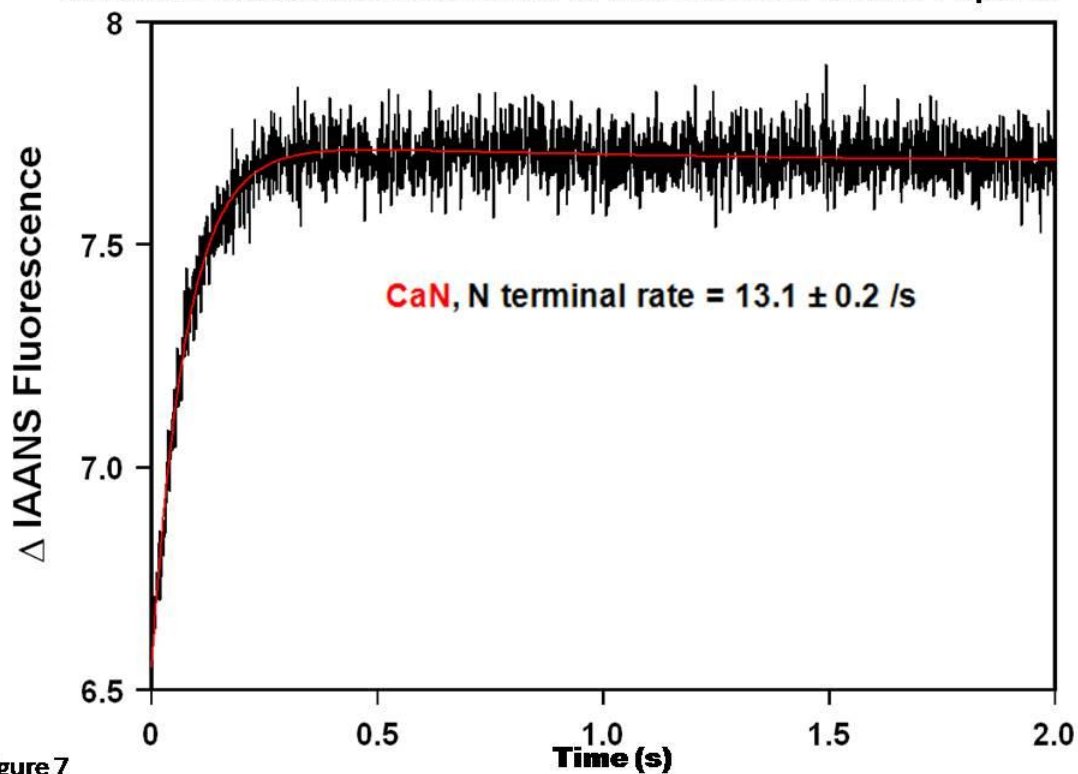


Figure 7

### Calcium Dissociation from CaM in the Presence of CKII Peptide

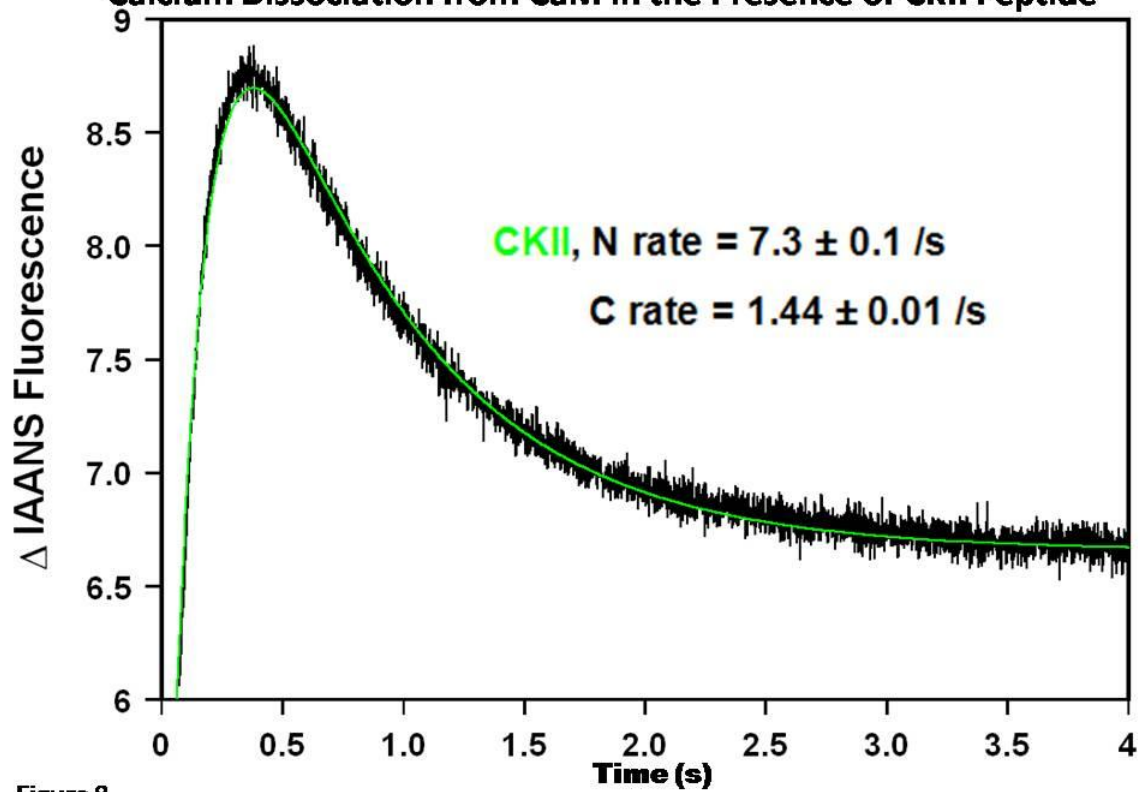


Figure 8

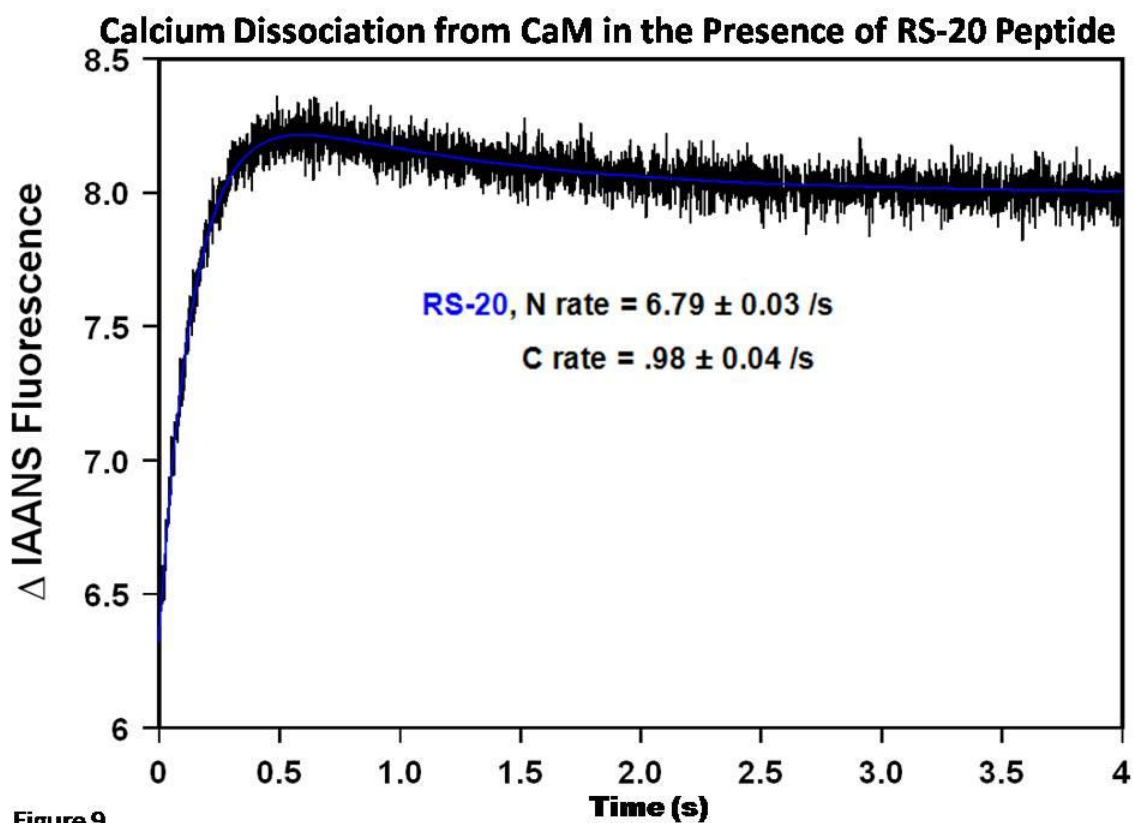


Figure 9

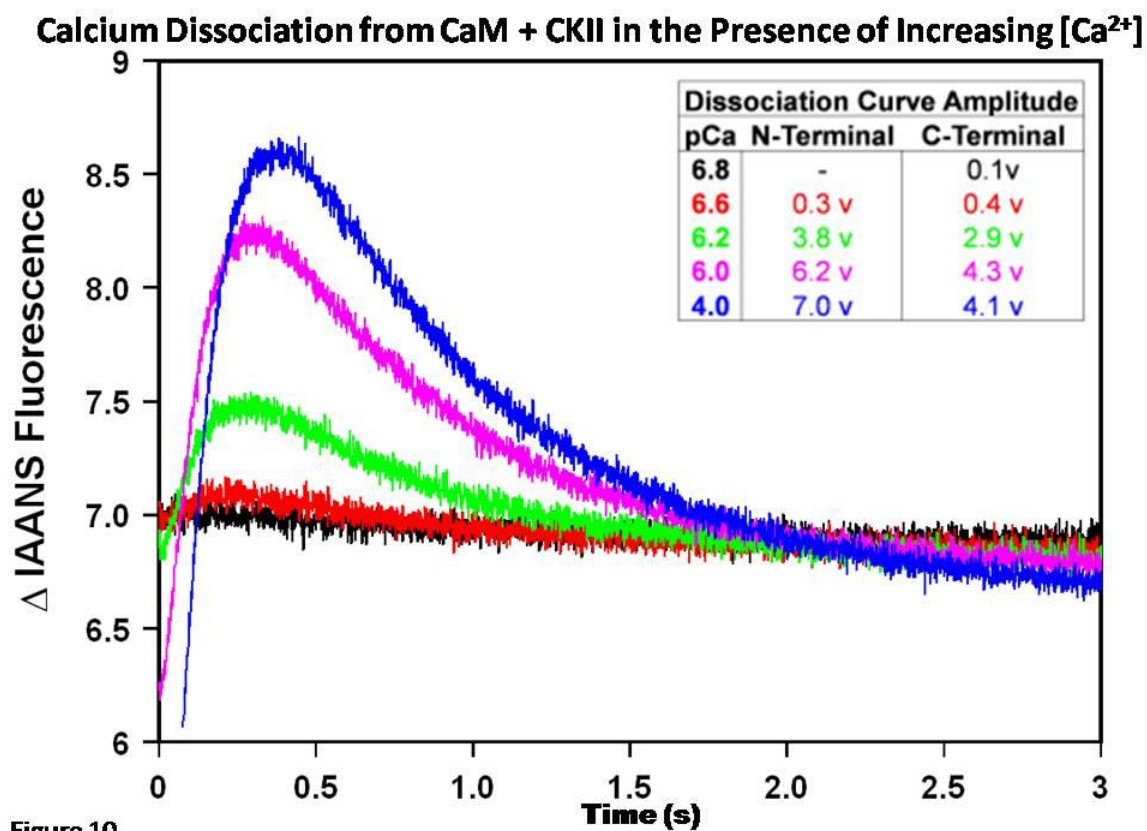


Figure 10



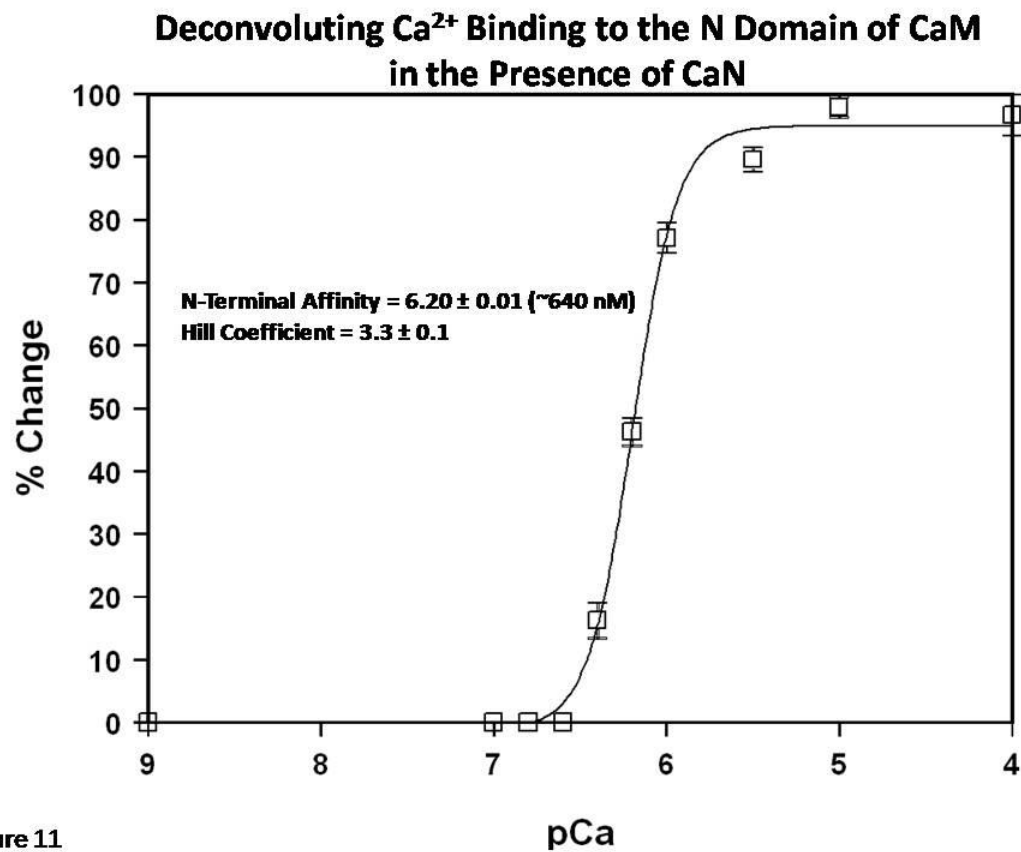


Figure 11

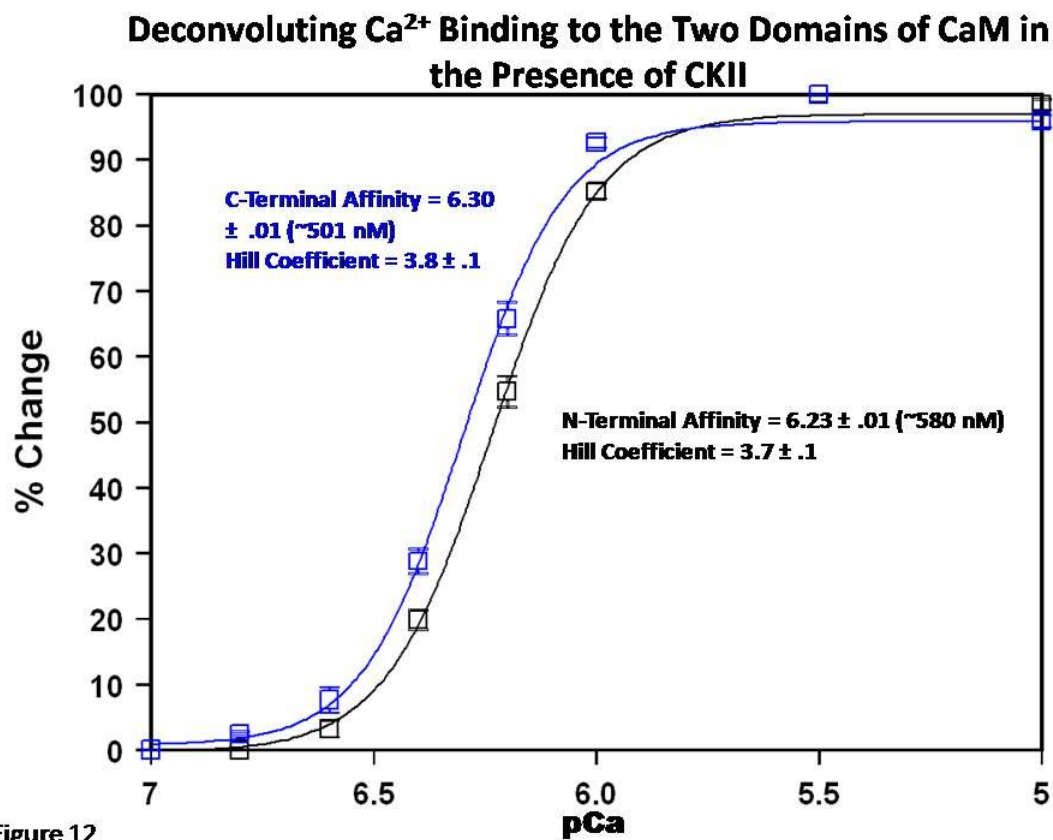


Figure 12

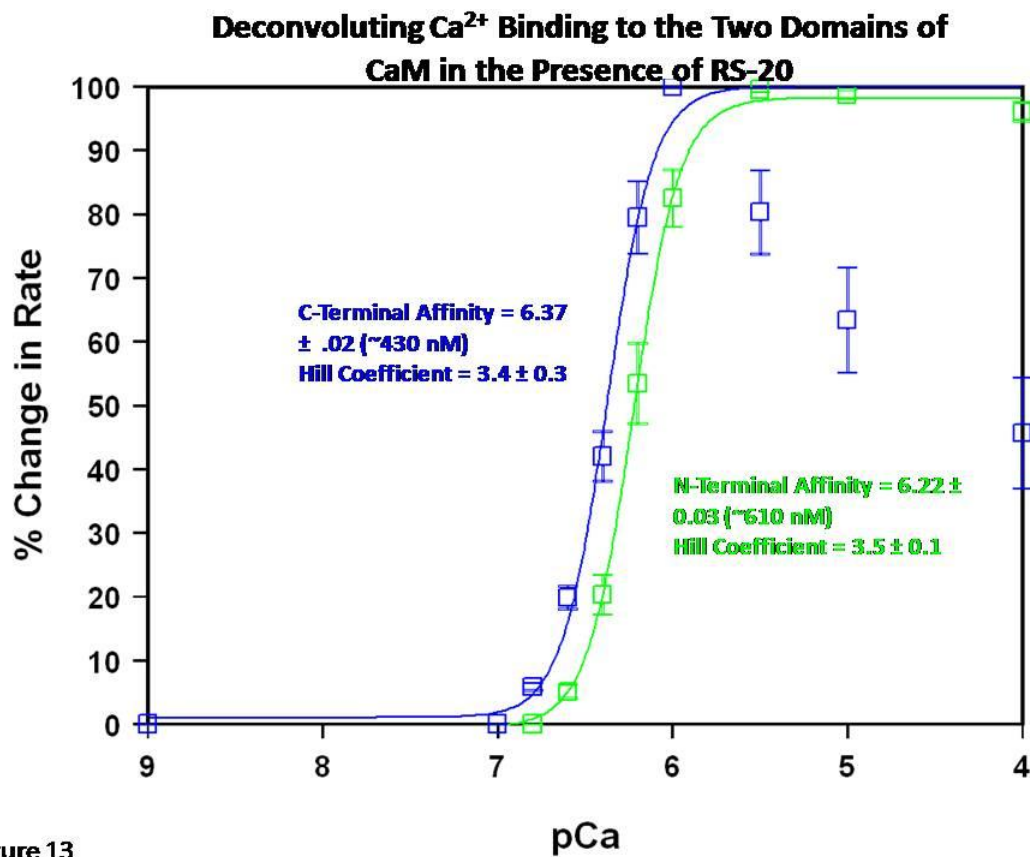


Figure 13

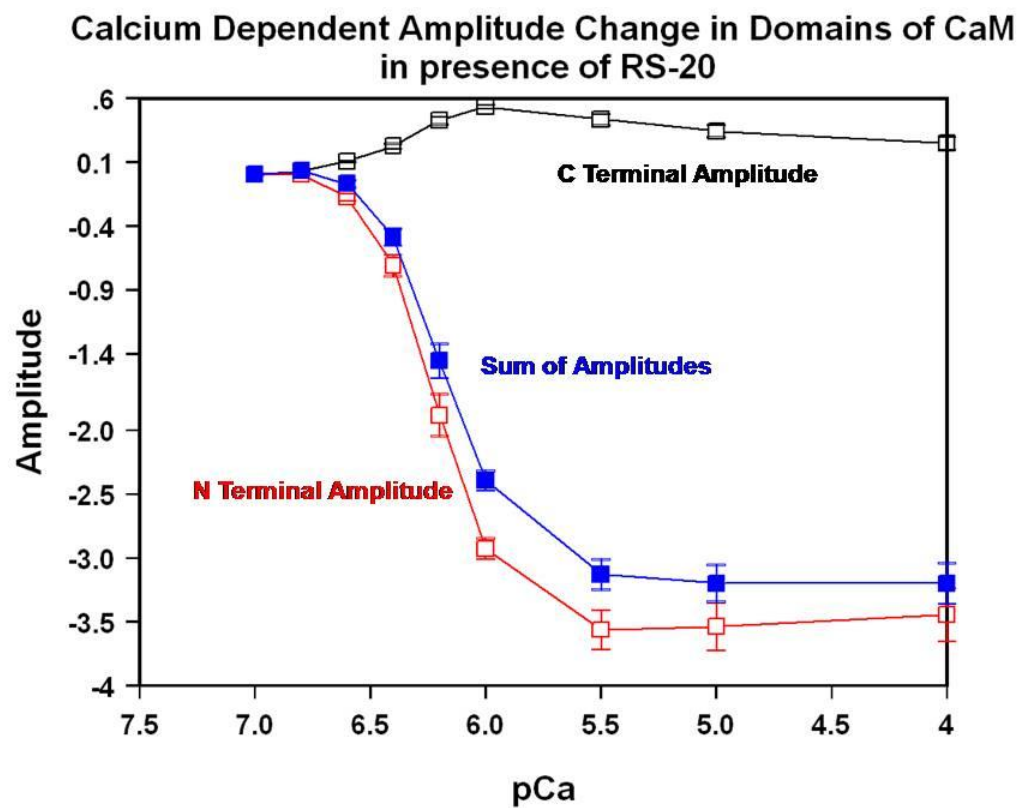


Figure 14

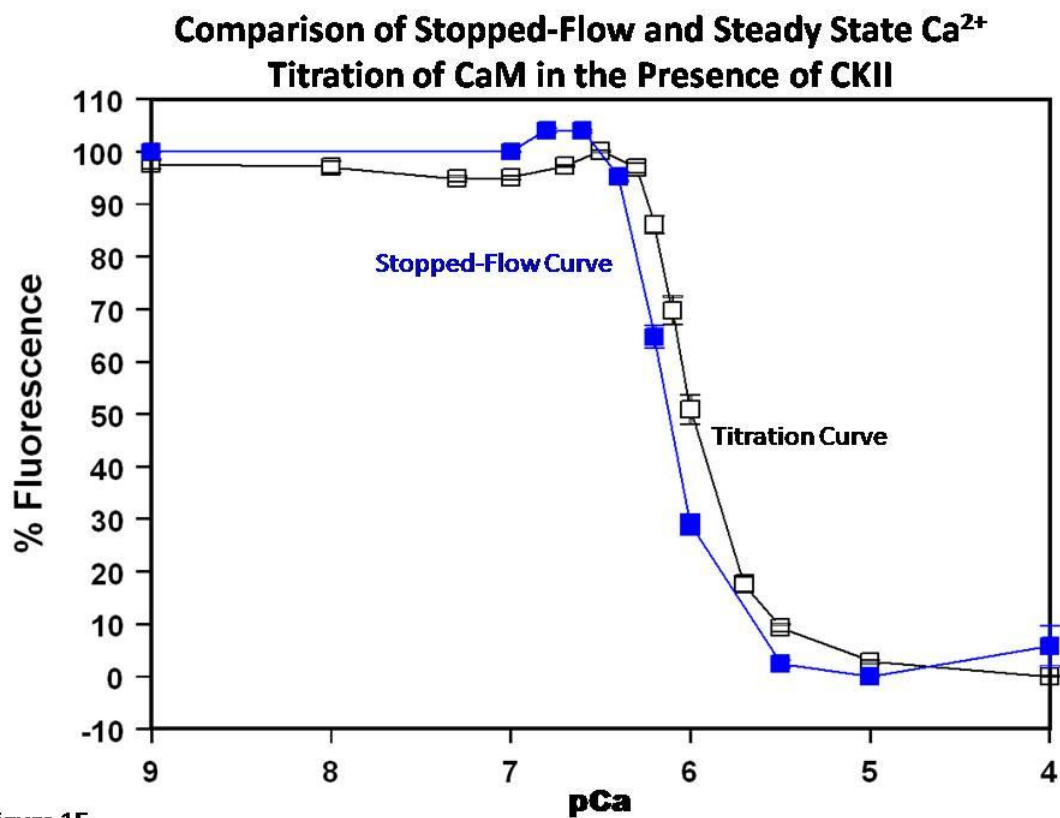


Figure 15

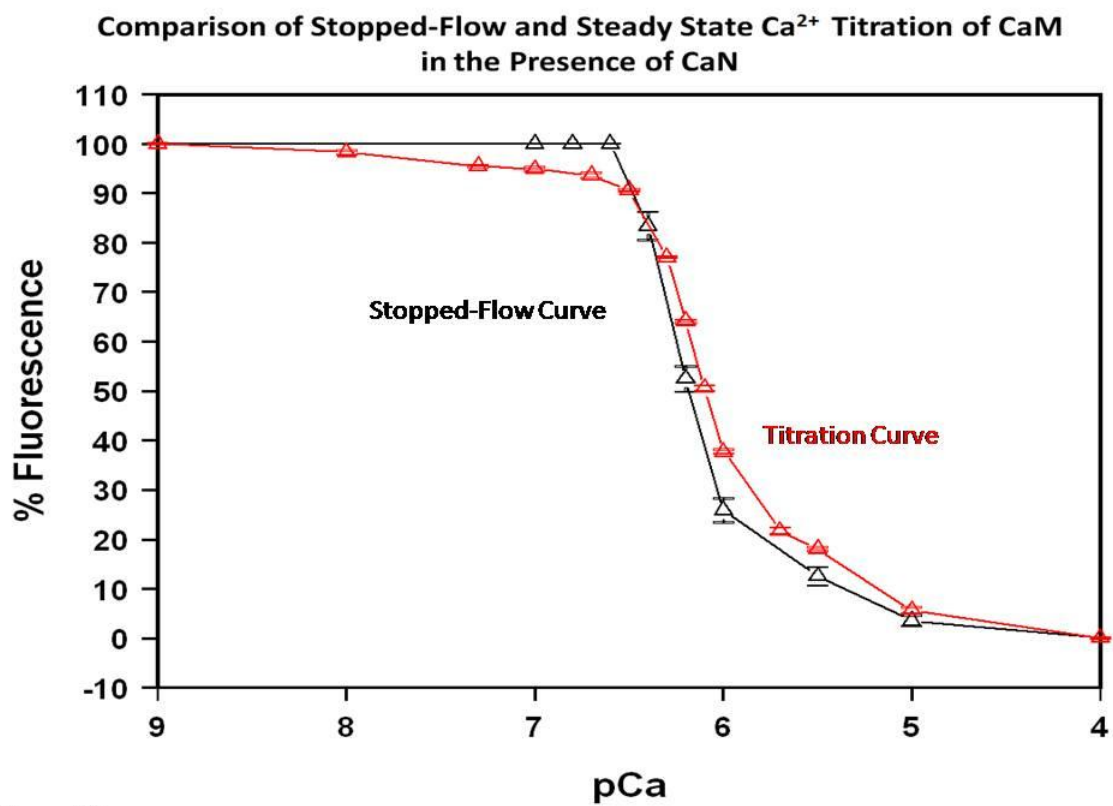


Figure 16

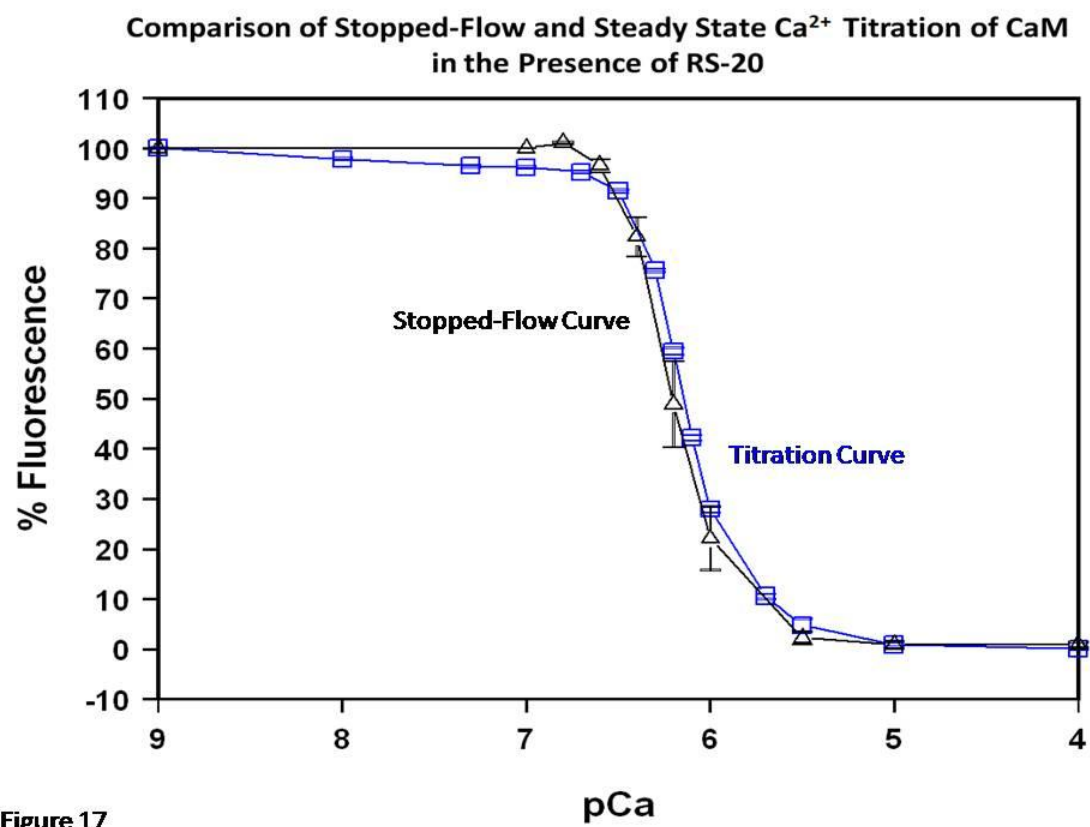


Figure 17